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^{113}Cd NMR as a Probe of the Active Sites of Metalloenzymes*

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^{113}Cd NMR has been used to study the active site metal ion(s) of the $^{113}\text{Cd}(\text{II})$ derivatives of four $\text{Zn}(\text{II})$ metalloenzymes, carboxypeptidase A, carbonic anhydrases, alkaline phosphatase, and superoxide dismutase. The resonances of the enzyme-bound $^{113}\text{Cd}(\text{II})$ ions are extremely sensitive to ligand exchange, including solvent and inhibitor, and to changes in the metal ion coordination sphere. The nature of this behavior can be shown to parallel the known structural properties and proposed roles of the metal ion in the catalytic mechanisms. Models accounting for the exchange mechanisms which may be modulating the chemical shift, linewidth, and coupling are discussed.

INTRODUCTION

NMR of metal nuclei has in general been too insensitive to be useful in the investigation of the molecular environment of metal ions bound at specific sites in biological macromolecules. We have recently shown that a modified Bruker HFX 90 spectrometer using a retuned ^{13}C probe operating at 19.96 MHz can achieve sufficient sensitivity that the NMR signals of the ^{113}Cd nucleus from $\text{Cd}(\text{II})$ bound at the position of the central metal ion in a number of metalloenzymes can now be used to explore the nature of the coordination complexes at the active sites (1). Zinc(II) metalloenzymes are widely distributed in nature (2). In most cases $\text{Cd}(\text{II})$ can be substituted for $\text{Zn}(\text{II})$ at the active site, often with retention of activity (2, 3). Thus $^{113}\text{Cd}(\text{II})$ with spin $\frac{1}{2}$ appears to be an ideal metal ion with which to carry out an investigation of the structure of these active sites by combining the NMR of both the central metal ion and the ligand nuclei. This paper summarizes the current state of our NMR investigations on a number of $^{113}\text{Cd}(\text{II})$ -substituted zinc metalloenzymes.

For most metalloenzymes, the metal ion is located at the active site and is directly involved in the catalytic process. The functional role of the metal ion covers a broad spectrum and includes activation of a nucleophile, direct coordination of substrate or product, and polarization of the scissile bond depending on the specific enzyme considered. In a few instances, however, the metal ion appears to play a purely

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structural role. The precise function of the metal ion must therefore be influenced by the surrounding polypeptide structure and the resulting constraints imposed on the coordination geometry of the metal ion. The nature of the coordination sphere of the metal ion and the rate(s) at which alterations in the immediate environment of the metal ion occur are fundamental to the mechanism of action of the enzyme. Thus an understanding of the static or average structure as well as dynamic fluctuations in the metal-ion binding site are essential in elucidating the detailed enzymatic mechanism.

The chemical shift range for common compounds of cadmium is over 600 ppm. Hence ^{113}Cd NMR is not only a particularly sensitive probe of alterations in the nature and coordination geometry of the ligands, but may also enable one to study processes modulating the resonances (e.g., ligand exchange) with lifetimes in the range of 10^{-1} to 10^{-5} sec. Thus, depending on the chemical shift difference between different coordination states, it may be possible to determine the relative exchange rates for ligands from solution, including solvent water.

MATERIALS AND METHODS

Bovine carbonic anhydrase B (BCAB) and human carbonic anhydrases B and C (HCAB, HCAC) were isolated from bovine and human erythrocytes, respectively (4). The apocarbonic anhydrases were prepared by removal of zinc by dialysis against *ortho*-phenanthroline (5) or pyridine-2,6-dicarboxylic acid (6).

Carboxypeptidase A (CPD) prepared according to Cox *et al.* (7) was obtained from Sigma Chemical Co. (St. Louis, Mo.), Lot No. 56C-8100. Apo CPD was prepared by dialyzing CPD (0.1 mM, 70 ml) against two changes of 1 liter of 5×10^{-3} M pyridine-2,6-dicarboxylic acid, 0.1 M Tris-HCl, 1 M NaCl, pH 7.6 at 4°C over a 2-day period. This was followed by dialysis against four changes of 1 liter of 0.1 M Tris-HCl, 1 M NaCl pH 7.6 at 4°C.

Alkaline phosphatase (AP) was isolated from *E. coli* CW 3747. Details of the method of isolation and preparation of the apoenzyme by treatment with Chelex 100 were as previously described (8).

The metal-free apoproteins (BCAB, HCAB, HCAC, CPD, and AP) were reconstituted by addition of 96 at% $^{113}\text{Cd}(\text{II})$ salts (Oak Ridge Laboratories) in the appropriate stoichiometry.

Superoxide dismutase (SOD) was prepared from bovine erythrocytes (9) and was freed of BCAB by affinity chromatography (10). The resulting SOD had an A_{259}/A_{280} ratio of 27.2. Aposuperoxide dismutase was prepared as previously described (11). The enzyme was reconstituted by successive additions of $^{113}\text{Cd}(\text{II})$ and spectrographically pure Cu(II) (12) (Johnson, Mathey and Co., London).

^{113}Cd FT NMR spectra were obtained on an FT Bruker HFX 90-MHz spectrometer with an operating frequency of 19.96 MHz modified for multinuclear capability in a manner similar to that previously described (13). D_2O , present as either the bulk solvent or in a 3-mm coaxial capillary insert, provided the deuterium lock for field-frequency stabilization. Data acquisition employed a spectral width of 5000 Hz with 1024 accumulated data points and a pulse repetition rate of 0.3–0.5 sec. The Fourier transform was carried out with 2K time-domain points providing a resolution of 5 Hz/point. A two-fold scale expansion was used for all spectra shown. Measurements were made at $25 \pm 2^\circ$ on ~ 1.0 -ml samples contained in 10-mm sample tubes.

Approximately 50,000 to 250,000 transients were required to obtain each spectrum depending on the protein concentration. Proton decoupling was not employed because of the negative value of the nuclear magnetic moment for ^{113}Cd and the predicted value of the negative NOE on correlation time. ^{113}Cd chemical shifts are expressed relative to the resonance position of 0.1 M $\text{Cd}(\text{ClO}_4)_2$.

RESULTS AND DISCUSSION

For the current study we have selected the $^{113}\text{Cd}(\text{II})$ derivatives of four Zn(II) metalloenzymes, alkaline phosphatase (AP), carboxypeptidase (CPD), carbonic anhydrases,¹ and superoxide dismutase (SOD), since they illustrate the broad range of reactions catalyzed by Zn(II) metalloenzymes. The first two enzymes catalyze hydrolyses of phosphate monoesters and peptide bonds, respectively. Carbonic anhydrase catalyzes the physiologically important hydration of CO_2 , while superoxide dismutase catalyzes the dismutation of the superoxide radical, O_2^- , to H_2O_2 and O_2 ; a protective reaction of physiological importance. The general features of the first coordination sphere of the metal ion at the active sites of these four enzymes are summarized in Scheme I. With the exception of alkaline phosphatase the information is derived from crystal structures at high resolution (14–16). The histidyl nitrogen ligands for alkaline phosphatase are deduced from the EPR spectra of the Cu(II) enzyme and data on the photooxidation of histidyl residues in the protein (17, 18).

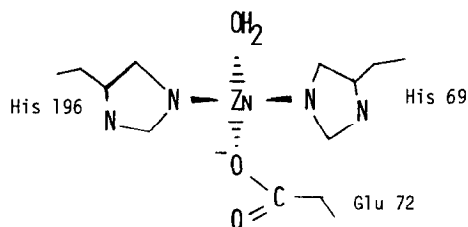
Substitution of Cd(II) for Zn(II) in these proteins results in enzymes which retain at least partial activity. Cadmium carboxypeptidase hydrolyzes the esters analogous to its natural peptide substrates even more rapidly than the native enzyme (19). Cadmium carbonic anhydrase (HCAB) is active on its ester substrates with a $\text{pK}_{\text{activity}}$ of 9.1 compared to 7.5 to 8 for the native enzyme (20). Cadmium alkaline phosphatase forms the normal intermediates on the reaction path, but formation and breakdown are slowed by at least 3 orders of magnitude (3, 8). Activity of superoxide dismutase is little affected by Cd(II) substitution (12).

Three of the enzymes appear to have at least one inner coordination site open for solvent interaction or binding of monodentate ligands from solution. The simplest picture is given in Scheme I, although 5-coordinate species with two monodentate ligands from solution are possible and may be involved in ligand exchange mechanisms (21). A stable 5-coordinate complex can be formed between carbonic anhydrase and two cyanide ligands (22). It is not known whether external ligands actually enter the inner coordination sphere of the metal ion in alkaline phosphatase. The mechanism may involve outer-sphere interactions exclusively. Solvent does not appear to have access to the IIB metal site of superoxide dismutase (23). The presence of this metal must modulate the structure of the Cu(II) site 6 Å away which interacts with O_2^- and shares the imidazole ring of His-61 as a ligand. The latter must be in the imidazolate anion form.

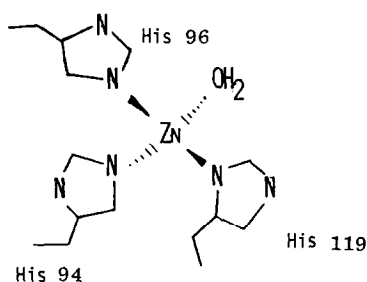
In some cases there is definitive evidence to indicate that variations in the nature and rate of exchange of monodentate ligands from solution as a function of pH and buffer concentration parallel the activity of the enzyme, e.g., carbonic anhydrase (24). Thus,

¹ Carbonic anhydrases reported on here are: human carbonic anhydrase B (HCAB), human carbonic anhydrase C (HCAC), and bovine carbonic anhydrase B (BCAB).

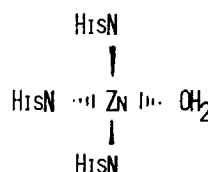
CARBOXYPEPTIDASE



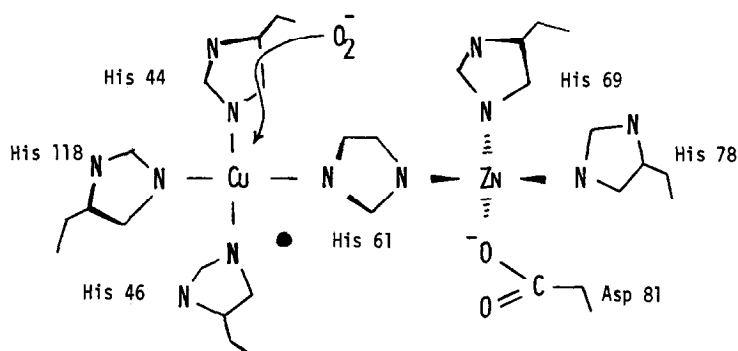
CARBONIC ANHYDRASE



ALKALINE PHOSPHATASE



SUPEROXIDE DISMUTASE



SCHEME I

the presence or absence of an observable ^{113}Cd resonance as a function of solution conditions (e.g., pH, buffer concentration) can be directly related to the rate of exchange processes occurring at the metal ion, which in turn reflects the efficiency of catalytic function.

The limited data which have been obtained to date do not permit a detailed analysis of the coexisting states in these systems or the lifetime of each state. Sufficient data do

exist, however, to allow interpretation of the results in terms of a two-state or two-conformer exchange model. Despite the simplifying assumptions required in the analysis of the data, the potential of this method in the determination of the limiting rates of exchange processes can be adequately demonstrated. Thus, even at this rudimentary level, these results can provide a criterion for the validity of proposed mechanisms for these metalloenzymes.

From initial considerations it was anticipated that the resonance from the enzyme-bound $^{113}\text{Cd(II)}$ ion should be slightly narrower than the resonance of a nonprotonated ^{13}C in the same protein due to the reduced magnetogyric ratio of the ^{113}Cd nucleus and the increased distance to abundant magnetic nuclei (e.g., protons). Therefore in the absence of other complications, such as a large contribution to the relaxation from chemical shift anisotropy, a resonance should be observed in both the slow and fast exchange limits. From a determination of the chemical shift difference ($\Delta\omega$) between two exchanging states the limits of exchange (slow to fast) can be defined. From the data of Table 1 (see Scheme II for proposed exchanging states) the magnitude of $\Delta\omega$ for the states of the enzyme-bound metal ion is ~ 50 – 100 ppm. Thus sufficient broadening

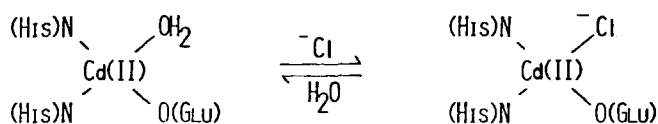
TABLE 1
 ^{113}Cd CHEMICAL SHIFTS FOR $^{113}\text{Cd(II)}$ -SUBSTITUTED Zn(II)
METALLOENZYMES

System ^a	pH	δ^b
CPD + β -phenylpropionate	6.9	133
BCAB	8.0	214
HCAC	8.1	225.7
HCAC + 4 eq Cl^-	8.1	239.0
HCAB	9.1	145.5
HCAB + 4 eq F^-	8.9	—
HCAB + 4 eq F^- + 2 eq I^-	8.9	225.5
HCAB + 4 eq Cl^-	8.9	241.0
HCAB + 4 eq Cl^- + 4 eq I^-	8.9	225.5
HCAB + 4 eq Br^-	8.9	236.2
HCAB + 4 eq I^-	8.9	225.5
AP	6.0	117.2
AP + F^-	6.0	123.0
AP + Cl^-	6.0	170.0
AP + Br^-	6.0	169.3
AP + I^-	6.0	169.5
AP + 1 eq P_i	6.5	55; 142
$\text{Cd(II)}_2\text{SOD}$	6.0	170.2
$\text{Cd(II)}_2\text{Cu(II)}_2\text{SOD}$	6.0	—
$\text{Cd(II)}_2\text{Cu(I)}_2\text{SOD}$	4.6	8.6
CdCl_2 (in standard buffer)	6.0	55.8

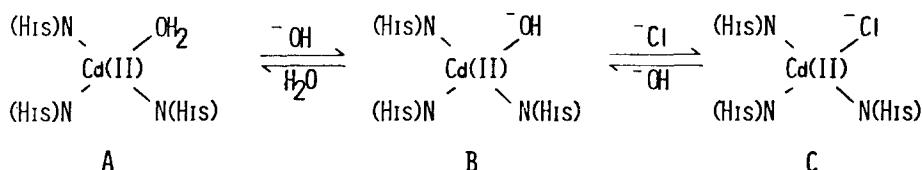
^a For buffer conditions and sample concentrations see the appropriate figure legends.

^b Parts per million from the external standard, $0.1\text{ M Cd}(\text{ClO}_4)_2$.

CARBOXYPEPTIDASE



CARBONIC ANHYDRASE



SCHEME II

of the signal to result in the apparent absence of a resonance under our operating conditions could occur when the lifetime, τ , of a particular state was within the limits $10^{-1} \geq \tau \geq 10^{-5}$ sec.

Carboxypeptidase A

Carboxypeptidase A is a pancreatic exopeptidase of 34,600 MW which removes the C-terminal amino acid residue from peptides and proteins. The enzyme works best if the side chain of the C-terminal residue is aromatic or branched aliphatic. The analogous synthetic esters are also hydrolyzed efficiently. The active enzyme is a monomer containing a single Zn(II) ion at the active site (2).

In the absence of inhibitor, no ^{113}Cd NMR resonance can be detected from a 4.6 mM solution of $^{113}\text{Cd(II)CPD}$ at pH 6.5 in 1M NaCl, 0.1 M Tris-HCl (Fig. 1A). Addition of 10 equivalents of the inhibitor β -phenylpropionate per mole of enzyme results in the appearance of a resonance at 132 ppm. Using ^{35}Cl NMR, Stephens and Bryant (25) have determined the mean residence time, τ , of Cl^- at the Cd(II) site in CPD to be 3×10^{-7} sec under similar pH and buffer conditions. According to our model this lifetime requires a chemical shift difference between the two states ($\pm \text{Cl}^-$) much greater than the entire range of chemical shifts for ^{113}Cd in order to account for the absence of an observed resonance in the uninhibited enzyme. Either the lifetime calculated from the ^{35}Cl NMR data is 2 to 3 orders of magnitude too short or there exists an additional contribution from two states of differing chemical shifts which have much longer lifetimes. This could arise from a slow fluctuation in the protein ligand coordination geometry at the metal ion or from a more remote effect such as a slow rate of exchange between two different conformational states of the protein. Both local fluctuation at the metal ion and interconversion of protein conformers may be slowed by inhibitor binding. One possible explanation for this may be the competition between tyrosine-248 and solvent ligand (Cl^- or H_2O) for metal coordination (26).

On three separate measurements of the ^{113}Cd spectrum of $^{113}\text{Cd(II)CPD} + \beta$ -phenyl-

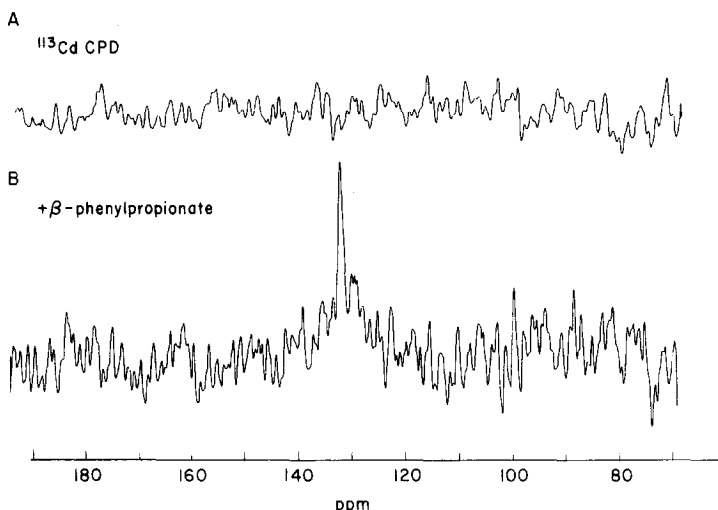


FIG. 1. ^{113}Cd NMR spectra of $^{113}\text{Cd}(\text{II})$ carboxypeptidase A (CPD). Conditions: 0.1 *M* Tris-chloride, 1.0 *M* NaCl. (A) 4.60×10^{-3} *M* $^{113}\text{Cd}(\text{II})\text{CPD}$, pH 6.53; (B) 4.60×10^{-3} *M* $^{113}\text{Cd}(\text{II})\text{CPD}$, 46.0×10^{-3} *M* β -phenylpropionate, pH 6.90.

propionate, asymmetry of the resonance or possible additional small resonances to high field of the major resonance were observed (Fig. 1B). These may represent a small proportion of the protein in a less favored conformer. This could also arise from the binding of excess inhibitor to the enzyme surface (27).

Carbonic Anhydrase

Carbonic anhydrase is ubiquitously distributed in both plants and animals. All appear to be $\text{Zn}(\text{II})$ metalloenzymes with molecular weights of $\sim 30,000$ (2). The most frequently studied enzymes are the two isozymes (B and C) from human erythrocytes. They catalyze the reversible hydration of CO_2 , probably using a zinc-coordinated hydroxide ion to effect the reaction, $\text{CO}_2 + ^-\text{OH} \rightleftharpoons \text{HCO}_3^-$, a reaction which is the major pathway of the uncatalyzed reaction only at very high pH values.

In the proposed reaction mechanism of carbonic anhydrase, a proton transfer step is obligatory (24). Buffer molecules have been shown to modulate the catalytic process by acting as proton acceptors and increasing the rate of proton transfer from the active site (28–30). Monovalent anions which may compete with hydroxide anion for an open coordination site on the metal ion have been shown to shift the apparent *pK* for activity (9.1 for $\text{Cd}(\text{II})\text{HCAB}$) to even higher pH. Models proposed for the catalytic mechanism assign the proton dissociation to the ionization of either a water molecule directly coordinated to the metal ion (24) or an adjacent histidyl residue.²

Assuming that the same ionizing group is functional in both the $\text{Cd}(\text{II})$ -substituted

² It has been suggested that the activity-linked ionization might be the ionization of the pyrrole NH on one of the histidyl ligands, the *pK_a* being lowered by the inductive effect of $\text{Zn}(\text{II})$ coordination (31). This hypothesis seems somewhat doubtful, since the C2 protons on the three-coordinated histidyl residues have been observed in the proton NMR spectra and they show little chemical shift change through the pH region of the activity-linked ionization (32).

enzyme and the native enzyme, the ^{113}Cd NMR study described below was undertaken in an attempt to distinguish between these two models. Halides are known inhibitors of carbonic anhydrase (33) and interact directly with the metal ion, as shown by ^{35}Cl and ^{81}Br NMR studies on the native enzyme (34–36) as well as by X-ray spectroscopy of these complexes (37). This fact is important, since at a pH below the pK of 9.1 and in the absence of an inhibitor the ^{113}Cd resonance may not be observable if states A and B, shown in Scheme II, have significantly different chemical shifts which can be exchange broadened depending on the lifetimes. The absence of ^{113}Cd resonance at pH 8.9 for $^{113}\text{Cd(II)HCAB}$ is in accord with this interpretation (Fig. 2A). It does not, however, necessarily distinguish between the identities of the ionizing groups, since ionization of an adjacent histidyl residue may also induce large perturbations in the ^{113}Cd chemical shift of active site $^{113}\text{Cd(II)}$.

The pK_a for esterase activity of the Zn, Mn, and Cd enzymes increases in the order $\text{Zn} < \text{Mn} < \text{Cd}$, i.e., the same order in which the ionic radius increases. This is in agreement with the ionized water model since a pK_a of 9.1 for Cd(II)HCAB appears to be too high for the "imidazole" model.

A sample of $^{113}\text{Cd(II)HCAB}$ to which 4 equivalents of F^- were added still shows no resonance (Fig. 2B). However, the addition of 4 equivalents of Cl^- , Br^- , and I^- at this same pH results in the appearance of a resonance with increasing S/N paralleling the order of the decreasing dissociation rate constants for this series. The chemical shift values for this series are given in Table 1. Thus exchange broadening is inhibited by the addition of halides at pH values below the pK . This is consistent with the two-state model ($A = B$, Scheme II) being effectively removed by the competition of halide with H_2O and OH^- at this pH. State C (Scheme II) would now be preferred with a lifetime $\tau \geq 10^{-2}$ sec corresponding to a slow exchange model for the halide. Contrary to the native Zn(II) enzyme, no ^{35}Cl NMR line broadening was observed for the Cd(II)HCAB . The proposed explanation of this result was an altered coordination geometry or saturation of the first coordination sphere by protein ligands (36). The ^{113}Cd results presented here demonstrate that the halides are interacting directly with the Cd(II) ion and are in slow exchange. Slow exchange would account for the absence of broadening in the ^{35}Cl NMR studies. On addition of I^- to the sample shown in Figs. 2B and C a resonance appears at the chemical shift value for $^{113}\text{Cd(II)HCAB} + 4 \text{ eq of } \text{I}^-$ (Fig. 2E), 225.5 ppm. This demonstrates a greater affinity of I^- for the enzyme compared to F^- and Cl^- and is consistent with a unique active site Cd(II) ion coordination for this series.

In discussing the relative stability of the halide complexes of carbonic anhydrase it should be recalled that the magnitude of the stability constants for simple Cl^- , Br^- , and I^- complexes increases by several orders of magnitude on substituting Cd(II) for Zn(II) and the order of stabilities changes from $\text{Cl}^- > \text{Br}^- > \text{I}^-$ to $\text{I}^- > \text{Br}^- > \text{Cl}^-$. For example, the equilibrium constant for ZnCl_4 is 1 compared to 10^3 for CdCl_4 ; 10^{-2} for ZnI_4 and 10^6 for CdI_4 (38). It is thus not surprising that while a given halide, Cl^- , e.g., may be in fast exchange for the Zn(II) enzyme, it may be in slow exchange for the Cd(II) enzyme. The stabilities of the halide complexes of Zn(II) carbonic anhydrase follow the series $\text{I}^- > \text{Br}^- > \text{Cl}^-$ paralleling the order for the Cd(II) halides, but opposite to that for the Zn(II) halides. This has been attributed to the particular environment of the Zn(II) site in the enzyme in a deep pocket in a relatively water-free region (39).

If the ionizing group with a pK_a of 7 in the native enzyme and a pK_a of 9.1 in the Cd(II)-substituted enzyme are one and the same, then the ^{113}Cd NMR results presented above suggest the identity of this group to be a metal-coordinated water molecule.

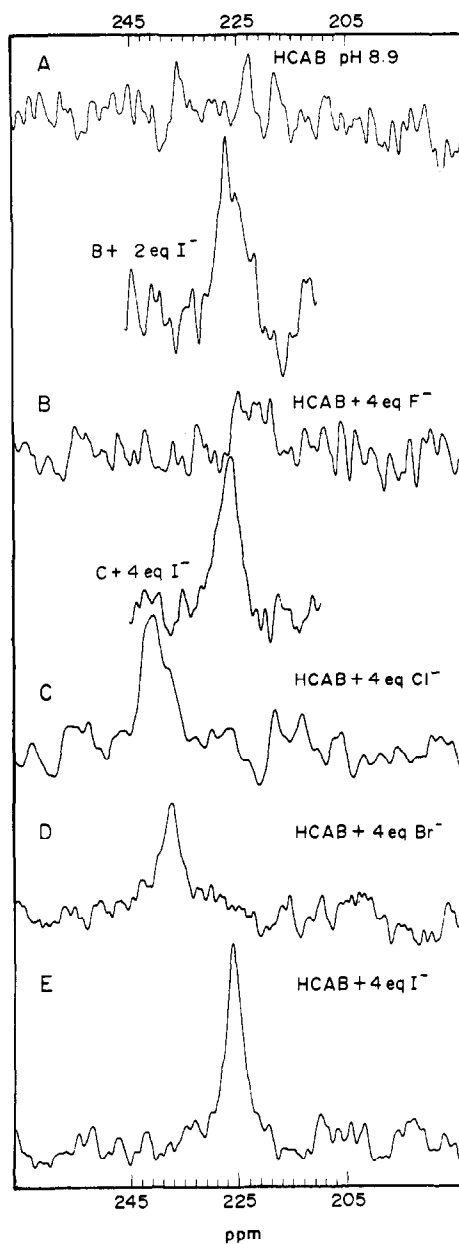


FIG. 2. ^{113}Cd NMR spectra of $^{113}\text{Cd(II)}$ human carbonic anhydrase B (HCAB). Effect of halide binding. Conditions: $6.13 \times 10^{-3} M$ $^{113}\text{Cd(II)HCAB}$, $0.025 M$ Tris-sulfate, pH 8.9 for all spectra with additions as indicated. (A) No halide present; (B) $2.45 \times 10^{-2} M$ NaF, *insert*, (B) plus $1.22 \times 10^{-2} M$ NaI; (C) $2.45 \times 10^{-2} M$ NaCl, *insert*, (C) plus $2.45 \times 10^{-2} M$ NaI; (D) $2.45 \times 10^{-2} M$ NaBr; (E) $2.45 \times 10^{-2} M$ NaI.

Comparison of Isozyme and Species Variants of Carbonic Anhydrase

Both of the isozymes of carbonic anhydrase from human red cells have similar molecular weights and tertiary structure as determined by X-ray crystallography (2). Although there are a number of differences in amino acid sequence between them, there are also large regions of homology in the primary structure, suggesting that the two isozymes arose from a gene duplication. Despite the apparent similarity in general molecular structure, the C isozyme is approximately tenfold more active than the B isozyme under conditions where the proton transfer step is not rate limiting, i.e., where buffer is the proton acceptor from the protonated or acid form of the enzyme (28–33). The enzyme from bovine red cells (BCAB) has a maximum catalytic rate which resembles HCAC. A ^{113}Cd NMR study of the resonances from these three Cd(II)-substituted isozymes shows some interesting differences.

The initial studies of the ^{113}Cd resonances of Cd(II) carbonic anhydrases revealed that the ability to observe the resonance depends critically on pH (1). In general for the enzyme without additional ligands from solution, a resonance appears at pH 8 or above and disappears at pH values much below 8. For example, a well-resolved resonance is present for HCAB at pH 9.1 (Fig. 3A), but is absent at pH 8.9 (Fig. 2A). We believe this behavior is attributable to both the ionization state and the rate of exchange of solvent ligands (H_2O and ^-OH) as well as the proton exchange ($\text{Cd}^-\text{OH} + ^+\text{H} \rightleftharpoons \text{CdOH}_2$) on the coordinated solvent molecule. The fact that once the resonance of $^{113}\text{Cd(II)}$ HCAB has disappeared as $[\text{H}^+]$ is increased, an observable resonance can be restored by the substitution of a slowly exchanging halide supports this view (Fig. 2).

The dynamics of the solvent exchange are complex, however, and the pH dependency of the resonance is under further investigation and can only be partially analyzed at present. In terms of the activity-linked ionization of the enzyme (presumably the $\text{CdOH}_2 \rightleftharpoons \text{Cd}^-\text{OH} + \text{H}^+$ equilibrium), pH 9.1 is the $\text{p}K_a$ for this transition as determined by the pH rate profile of esterase activity and the pH dependency of the angular correlation of γ rays (20). Thus the resonance at 145 ppm for HCAB at pH 9.1 represents a 50:50 mixture of the acid and alkaline forms of the enzyme. One would conclude therefore that there is rapid exchange between these forms (i.e., rapid proton transfer) and that 145 ppm represents the average resonance position in the fast exchange limit. It would appear that the pH must be at or above the $\text{p}K_a$ for the proton transfer(s) to be sufficiently rapid for the fast exchange condition to apply.

The $\text{p}K_a$ for the activity-linked ionization of Zn(II)HCAC is ~ 1 pH unit lower than that for Zn(II)HCAB. Indeed the resonance for Cd(II)HCAC can be observed for ~ 1 pH unit below the point where the resonance for Cd(II)HCAB has disappeared, suggesting that this differential is maintained in the Cd(II) derivatives and further suggesting that the proton equilibria connected with the activity-linked ionization are modulating the $^{113}\text{Cd(II)}$ resonances.

At or near the presumed $\text{p}K_a$ for the activity-linked group of the three isozymes, the average chemical shifts of the resonances for the species in rapid exchange are considerably different: 145 ppm for HCAB, 214 ppm for BCAB, and 225 ppm for HCAC. These differences may reflect subtle changes in the coordination geometry of the protein ligands (three imidazole nitrogens), the presence of different adjacent amino acid side chains in the active site cavities, or the presence of different hydrogen bond

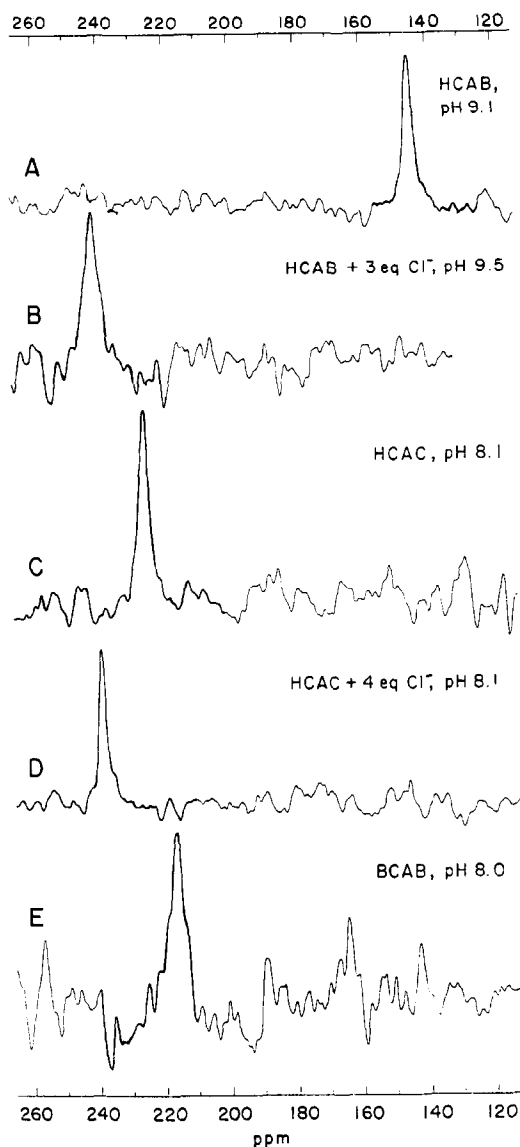


FIG. 3. ^{113}Cd NMR spectra of $^{113}\text{Cd}(\text{II})$ carbonic anhydrases. Conditions: 0.025 *M* Tris-sulfate. (A) 7.00×10^{-3} *M* $^{113}\text{Cd}(\text{II})\text{HCAB}$, pH 9.1; (B) 7.16×10^{-3} *M* $^{113}\text{Cd}(\text{II})\text{HCAB}$, 2.15×10^{-2} *M* NaCl, pH 9.5; (C) 1.00×10^{-2} *M* $^{113}\text{Cd}(\text{II})\text{HCAC}$, 4.00×10^{-2} *M* NaCl, pH 8.1; (E) 3.04×10^{-3} *M* $^{113}\text{Cd}(\text{II})\text{BCAB}$.

networks at each active site in which the metal-liganded solvent molecule participates. When proton transfer is not rate limiting, the C isozyme is approximately ten-fold more active than the B, and some other step, possibly ligand (HCO_3^-) exchange, is rate limiting. These differences in metal ion environment for the three isozymes revealed by the ^{113}Cd NMR may reflect the differences in bonding which alter such variables as ligand exchange rates.

One of the difficulties in a simple analysis of the processes modulating the ^{113}Cd

signal from the enzyme is that each isozyme contains a unique array of protonatable amino acid side chains with differing pK_a 's in the vicinity of the metal ion. These side chains are not sufficiently close to the metal ion to be ligated, but possibly close enough that proton exchange at these sites could alter the NMR signal from the metal nucleus. It is perhaps not surprising that the Cl^- complexes of each isozyme, complexes which are relatively more rigid, since they contain more tightly bound slowly exchanging external ligands, all have very similar chemical shifts for the ^{113}Cd resonances (Table 1).

Alkaline Phosphatase

Alkaline phosphatase is a dimeric Zn(II) metalloenzyme of MW 86,000 composed of two identical subunits each containing one active site Zn(II) ion. Other metal ion binding sites exist (AP as isolated contains 4 g at Zn(II) and 1–2 g at Mg(II) per mole of enzyme dimer (40)), but little is known about their structural positions in the protein. Using NMR, the chemical nature of the phosphate complexes of native and metal-ion-substituted analogs of alkaline phosphatase has been investigated (41–43). These studies have provided extensive data concerning the nature and rates of formation of the phosphate intermediates, the stoichiometry of phosphate binding, and the role of the metal ions in this enzyme. Since only two metal ions are required for activity much of the ^{31}P NMR and all of the ^{113}Cd NMR studies reported here have been carried out on enzyme samples that were first made metal free and therefore phosphate free, since phosphate binding is dependent on the metal ion (8). Apoenzyme samples thus obtained were reconstituted with 2 equivalents of $^{113}Cd(II)$ per mole of enzyme dimer. All ^{31}P NMR studies employing the buffer system 0.01 *M* Tris, 0.01 *M* NaOAc, 0.1 *M* NaCl are consistent with the specific binding of 1 equivalent of P_i per mole of enzyme dimer as either the noncovalent or the covalent complex. The binding stoichiometry suggests the presence of negative homotropic interactions between the subunits of the enzyme. ^{113}Cd NMR provides a direct and unambiguous method of demonstrating the presence of the postulated cooperativity since both active centers can be simultaneously observed on ligand interaction at a single site. Furthermore, ^{113}Cd NMR allows the assessment of the influence of buffer and salt conditions on changes in protein conformation of catalytic significance arising from changes in ligand interactions at the metal ion.

The ^{113}Cd NMR spectrum of $^{113}Cd_2AP$ shows a single resonance at 117.2 ppm (Fig. 4A). Thus the two active site Cd(II) ions in the unliganded protein dimer experience identical environments, supporting the X-ray data which shows that there is twofold symmetry between the monomers (44). Addition of 0.1 *M* NaF to the above sample gives rise to a resonance at 123 ppm (Fig. 4B). The magnitude of the chemical shift difference (~ 5 ppm) from that in the absence of halide suggests that there is only a small perturbation at the active site $^{113}Cd(II)$ ion. In the presence of each of the halides Cl^- , Br^- , and I^- at 0.1 *M* concentrations, a single resonance is again observed with identical chemical shift values of 170 ppm.

Two studies of halide binding to alkaline phosphatase by ^{35}Cl NMR show no increase in the linewidth of the ^{35}Cl resonance in the presence of the two Zn(II) or two Cd(II) enzymes (45, 46). Based, in part, on differences in the ^{35}Cl linewidth at higher metal ion stoichiometries, these results have been interpreted as indicating either the absence of a direct interaction of the halide ions at the metal ion (46) or the presence of bound chloride in slow exchange with bulk halide (45). On the basis of the ^{113}Cd NMR data,

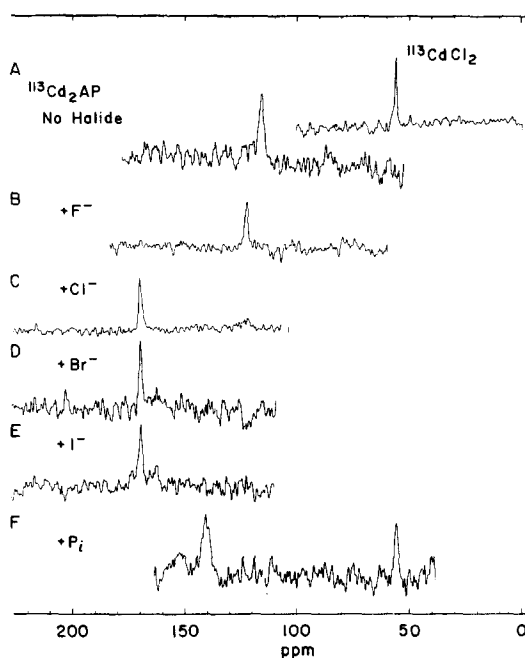


FIG. 4. ^{113}Cd NMR spectra of $^{113}\text{Cd}(\text{II})_2$ alkaline phosphatase. Conditions: 0.01 M Tris, 0.01 M NaOAc, $3.60 \times 10^{-3} M$ $^{113}\text{Cd}(\text{II})_2$ alkaline phosphatase, pH 6.0 for all spectra with additions as indicated. Spectrum of $^{113}\text{CdCl}_2$ (insert to spectrum A) was obtained under the same sampling conditions employed for the protein spectra; $3.00 \times 10^{-2} M$ $^{113}\text{CdCl}_2$, 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.0. (A) No halide present; (B) 0.1 M NaF; (C) 0.1 M NaCl; (D) 0.1 M NaBr; (E) 0.1 M NaI; (F) $4.5 \times 10^{-3} M$ $^{113}\text{Cd}(\text{II})_2$ alkaline phosphatase, $4.05 \times 10^{-3} M$ K_2HPO_4 , 0.1 M NaCl, pH 6.5.

the former of these possibilities appears more probable. Slow exchange implies a lower limit for the lifetime, τ , of bound Cl^- which is greater than 2×10^{-4} sec on the basis of the 50-ppm chemical shift difference for the ^{113}Cd resonance in the presence and absence of Cl^- . From the observed linewidth the value for τ must be of the order of 0.5×10^{-1} sec, which would be unreasonably long. In addition, and in contrast to the ^{113}Cd NMR of halide binding to HCAB, the observed chemical shifts for the series Cl^- to I^- in AP are identical, as are the linewidths. In HCAB the different chemical shifts and linewidths are evidence for slow exchange of halide within the first coordination sphere of the $\text{Cd}(\text{II})$ ion. Therefore a general conformational change in the protein structure induced by a protein-bound, but rapidly exchanging, halide is the most reasonable explanation for the observed chemical shift. Experiments to test this postulate directly by monitoring the effect of halide concentration and temperature on the ^{113}Cd resonance are currently in progress.

Phosphorylation of $^{113}\text{Cd}(\text{II})$ Alkaline Phosphatase

On addition of 1 equivalent of phosphate to AP at pH 6.5 (0.1 M NaCl), phosphorylation of the protein occurs at serine-99 and is reflected in the appearance of a single resonance in the ^{31}P NMR spectrum at 8.07 ppm (relative to 85% H_3PO_4) (41).

On phosphorylation, the ^{113}Cd resonance of $\text{Cd(II)}_2\text{AP}$ (Fig. 4C) splits into two resonances, each with an amplitude accounting for half the Cd(II) present, appearing at 142 and 55 ppm, respectively (Fig. 4F). A second equivalent of phosphate shows a ^{31}P resonance at 2.00 ppm (the chemical shift of free phosphate) and the ^{113}Cd NMR spectrum is not altered. Thus phosphorylation at a single site of the enzyme destroys the symmetric relationship between the metal-ion binding sites and simultaneously alters the environment of both Cd(II) ions from that present in the absence of phosphate. Phosphate in excess of one equivalent does not alter these initial changes. These observations are only consistent with the existence of negative homotropic interactions existing between the subunits.

Unequivocal assignment of the ^{113}Cd resonances in Fig. 4F to the phosphorylated and nonphosphorylated sites of the Cd(II) enzyme has not been made. However, the large difference in the resonance linewidths indicates that marked physical differences between the two sites in the phosphorylated enzyme must exist. The stability and high equilibrium concentration of the phosphoryl intermediate (E-P) in the Cd(II)_2 enzyme derive from an extreme slowing of the dephosphorylation rate relative to the phosphorylation rate on substitution of Cd(II) for Zn(II) . The dephosphorylation rate is $<0.01 \text{ sec}^{-1}$, which implies a lifetime, τ , for the E-P complex of $>100 \text{ sec}$. This lifetime is much longer than the reciprocal of the chemical shift difference and should give rise to two distinct resonances with linewidths similar to that of the unphosphorylated enzyme (Fig. 4F). Since E-P goes on to hydrolyze and form the noncovalent complex $\text{E} \cdot \text{P}$ (albeit slowly for the Cd(II) enzyme at pH 6.5, much more rapidly at alkaline pH), there must be solvent H_2O access to the phosphorylated site. The stoichiometric binding of phosphate which induces asymmetry in the initially symmetrical metal ion sites is presumably associated with conformational changes propagated across the monomer/monomer interface. This phenomenon may result in an alteration and stabilization of the structure at the unliganded metal ion site, making it inaccessible to both phosphate and solvent. If this speculation is correct then the resonance at 142 ppm can be assigned to this Cd(II) ion, as reflected in the increased linewidth of this resonance over the resonance at 55 ppm as well as the initial resonance at 170 ppm. Broadening of the resonance at 142 ppm would then be attributed to the appearance of proton coupling from coordinating ligands which must have lifetimes at the metal ion longer than the reciprocal of the coupling constant (e.g., for $J_{^{113}\text{Cd-H}} \sim 50 \text{ Hz}$, $\tau \geq 0.02 \text{ sec}$). This would be compatible with the exclusion of rapidly exchanging water molecules at this site. Such a hypothesis must be considered highly speculative, since no information exists about the actual molecular changes in the dimer which give rise to the negative cooperativity and the ability to phosphorylate only one site at a time (49). One might postulate the reverse of the picture given above; namely, that phosphorylation restricts access of the solvent to the metal ion and hence broadens the resonance. Since water is the nucleophile attacking the phosphorus of the phosphoserine, the former postulate seems perhaps more attractive.

Superoxide Dismutase

Superoxide dismutase from mammalian erythrocytes is a dimer of MW 31,300 composed of identical subunits each containing one ion of Cu(II) and Zn(II) . The crystal structure of this enzyme as determined by X-ray diffraction suggests that the

Zn(II) site is not accessible to solvent, while the copper site (6 Å away from the Zn(II)) is available to solvent from the direction above the plane of the ligands and appears to be the site of interaction with O_2^- (16, 48). Native metal ions were removed and the enzyme was reconstituted with two equivalents of ^{113}Cd per dimer following the procedure previously described (11). The $^{113}\text{Cd(II)}$ ions added bind selectively at the Zn(II) sites (11). ^{113}Cd NMR on this sample shows a single resonance at 170.2 ppm (Fig. 5A). This result is consistent with data indicating that the Zn(II) binding sites on each subunit are identical. Addition of two equivalents of Cu(II) to the above sample results in the complete disappearance of the resonance at 170.2 ppm (Fig. 5B). This result is expected, since a large paramagnetic contribution to the ^{113}Cd relaxation will

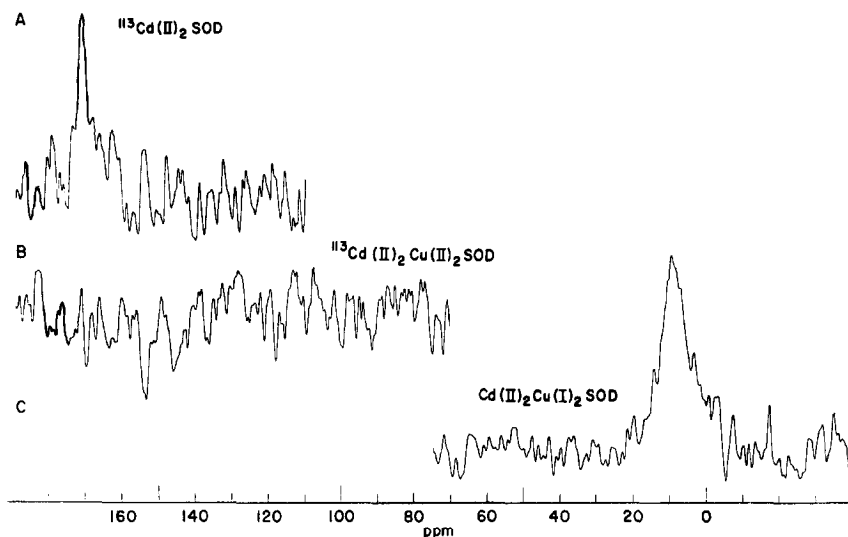


FIG. 5. ^{113}Cd NMR spectra of $^{113}\text{Cd(II)}$ superoxide dismutases (SOD). (A) $1.80 \times 10^{-3} M$ $^{113}\text{Cd(II)}_2$ SOD, D_2O , pH 6.0; (B) as (A) plus $3.60 \times 10^{-3} M$ Cu(II); (C) as (B) plus $6.20 \times 10^{-3} M$ $\text{Na}_2\text{S}_2\text{O}_4$, pH 4.6.

result from the relatively long electron spin–lattice relaxation time ($T_{1e} \sim 10^{-9}$ sec) of Cu(II) coordinated to the bridging imidazole ring. When the protein is exposed to $\text{Na}_2\text{S}_2\text{O}_4$, which reduces Cu(II) to Cu(I), a broad resonance reappears at 8.6 ppm. This large shift (~ 160 ppm) to high field is consistent with a $^{113}\text{Cd(II)}$ ion in a more symmetrical environment, perhaps resulting from the proposed release of His-61 by copper when the protein is reduced (48,49). The resulting protonation of His-61 would then remove the anionic charge present with both metals coordinated, increasing electron donation to the $^{113}\text{Cd(II)}$ ion. There are several possible mechanisms which could account for the magnitude of the apparent linewidth of this resonance. It cannot be attributed to a static paramagnetic contribution due to a small amount of Cu(II) since we observe no resonance in the unreduced protein (Fig. 5B). It may, however, indirectly result from the electron exchange with a small amount of Cu(II) enzyme remaining in the sample. Such a mechanism is compatible with the proposed mechanism

of action of this protein which includes a $\text{Cu(II)} = \text{Cu(I)}$ electron shuttle (50). Alternatively the broadening may be due to slight conformational fluctuations at the Cu(I) site enhance on release of His-61 and experienced by the ^{113}Cd ion giving rise to small chemical shift. What would be then observed is a resonance broadened in proportion to the rate of these fluctuations at the Cu(I) site. Other possibilities which cannot be ruled out are asymmetry of the subunits resulting in nonequivalence of the Cd(II) sites. Unresolved spin coupling to ligand protons is also possible, since this may be one of the most rigid sites among this set of enzymes (see exchange discussion below).

Exchange Contributions to the ^{113}Cd Resonance

In addition to the structural information which is available from a study of the ^{113}Cd NMR of Cd(II)-substituted metalloenzymes, this technique has provided a unique and extremely sensitive probe of the dynamic properties of the protein and solvent ligands which perturb the bound ^{113}Cd resonance. This is a consequence of the sensitivity of the ^{113}Cd chemical shifts to changes in both the geometry and the nature of coordinating ligands, similar to the large range in chemical shifts observed for other heavy metal nuclei, but amplified in the case of ^{113}Cd due to the large magnetogyric ratio for this metal ion, as reflected in a resonance frequency similar to that of ^{13}C . The sensitivity is further enhanced by the susceptibility of the heavier metals to alterations in valence shell electronic distributions.

The larger the chemical shift difference between the resonances of the Cd(II) ion in the exchanging states under investigation, the shorter the lifetimes required to satisfy both the slow and the fast exchange condition. With the inherent sensitivity of ^{113}Cd on our instrument for the dilute protein solutions employed, lifetimes much shorter than ~ 0.01 sec in the slow exchange limit will result in excess broadening which may be sufficient to abolish the ^{113}Cd resonance completely. The absence of a resonance for CPD in the absence of an inhibitor may reflect such a situation (Fig. 1A).

In Scheme II we have depicted models which may be representative of the coexisting states in these enzyme systems. All models involve an alteration in the nature of the metal ligands which would be expected to produce large changes in the ^{113}Cd chemical shift. In the case of Cd(II)HCAB, the proposed model accounts for a chemical shift difference of approximately 100 ppm. ^{113}Cd chemical shifts may also result from changes in protein conformation which do not alter the nature of the coordinating ligands, but perturb the coordination geometry of the metal ion. Such a change in ligand geometry is thought to give rise to the 160 ppm chemical shift difference observed between $\text{Cd(II)}_2\text{SOD}$ and $\text{Cd(II)}_2\text{Cu(I)}_2\text{SOD}$ (Figs. 5A and C). Additional experimental data are required to determine the magnitude of the ^{113}Cd chemical shifts possible in these latter circumstances, e.g., distorted tetrahedral versus tetrahedral or 5-coordinate complexes.

The observation of proton coupling to the ^{113}Cd nucleus from protein ligands or bound inhibitors in these metalloenzymes places even more stringent limits on the exchange rates which are modulating the chemical shift. In general, coupling constants will be 1 to 2 orders of magnitude less than the chemical shift difference for the Cd(II) ion in two coexisting states. Therefore lifetimes must also increase by the same order of magnitude. Stated simply, exchange may be slow from a chemical shift standpoint but

fast compared to the reciprocal of the coupling constant. For example, the greater instability of the active site metal complex in CPD implied by the shorter half-life of the Cd(II) ion in the active site of CPD as compared to HCAB (51) may be sufficient to abolish coupling and give rise to a much narrower resonance for Cd(II)CPD, as is observed (compare Figs. 1B and 3A). The Cd(II) site in $^{113}\text{Cd(II)}_2\text{Cu(I)}_2\text{SOD}$, on the other hand, gives rise to a resonance at 8.6 ppm indicative of a symmetrical ligand environment. The resonance linewidth is ~ 100 Hz and may result from coupling to ligand nuclei in a stabilized complex.

In a series of ^{113}Cd NMR studies of model complexes with amino acids, no proton spin coupling was observed. We have attributed this to the instability of the complexes at the pH values employed. We have observed proton coupling in complexes of $^{113}\text{Cd(II)}$ with EDTA at pH values ≥ 8 . From the formation constants for the 1:1 complex a lifetime on the order of 10^7 can be calculated. This exceeds by several orders of magnitude the lifetime required to abolish coupling in this complex. It has recently been suggested that resonance of protein-bound $^{113}\text{Cd(II)}$ ions are unlikely to have linewidths less than ~ 45 Hz (52). This is not a generally applicable statement, since the linewidth depends critically on the presence of coupling which can be modulated by the chemical exchange processes. In the absence of significant coupling, linewidths for ^{113}Cd bound to proteins of moderate size with only dipolar contributions to T_2 will be of the order of 20 Hz.

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